

Transcriptome analysis of paradormancy release in root buds of leafy spurge (*Euphorbia esula*)

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Leafy spurge is an economically important perennial weed that causes substantial losses to ranchers, farmers, and land managers because of reduced grazing and control costs. Although biocontrol agents for controlling leafy spurge have been effective in some areas, in most places, herbicide treatments or selected grazing by goats and sheep are still the primary means of control. The latter often proves ineffective for long-term eradication because leafy spurge is capable of reproducing through the development and maintenance of adventitious shoot buds located on the lateral roots of the plant (often referred to as root buds). Once formed, the buds enter a paradormant state and remain viable until they are released from dormancy by loss of the aerial portion of the plant. Various studies have indicated that the growing aerial shoot produces two signals that inhibit the growth of leafy spurge root buds (Horvath 1998, 1999; Horvath et al. 2002). One signal appears to be auxin produced in the growing shoot apices and young leaves, and the other signal appears to be sugar produced in the mature photosynthesizing leaves, which acts through gibberellic acid (GA) and abscisic acid signaling pathways (Chao et al. 2006; Horvath 1999). The leaf-derived signal appears to act through the plant hormone GA to inhibit cell division in the root buds by blocking the G1-to-S phase of the cell cycle (Horvath et al. 2002, 2005). The auxin signal likely acts indirectly (Cline 1991). Recently, orthologous carotene dioxygenases encoded by *RMS1* from pea (*Pisum sativum*), *MAX4* from arabidopsis (*Arabidopsis thaliana*), and *DAD1* from petunia (*Petunia hybrida*) have been shown to play a central role in auxin perception and correlative inhibition (paradormancy)

Bud dormancy is the primary mechanism by which the many perennial weeds escape herbicidal and mechanical control. We developed a 2,654-element Euphorbiaceae cDNA microarray using 1,886 sequenced cDNAs from the model perennial weed leafy spurge, 384 cDNAs from cassava, and 384 control genes from other plant, animal, and bacterial species. This array was used to follow changes in gene expression in root buds of leafy spurge following loss of paradormancy. The differential expression of several genes previously identified as being induced following loss of paradormancy was confirmed by microarray analysis. In addition, genes encoding an asparagine synthase, a phosphate-inducible protein, and a curculin-like (mannose-binding) lectin family protein were found to be rapidly up-regulated upon loss of paradormancy. Several genes involved in flavonoid biosynthesis were found to be rapidly down-regulated upon loss of paradormancy. The potential impact of flavonoid biosynthesis on auxin transport in response to bud growth is discussed.

Nomenclature: Leafy spurge, *Euphorbia esula* L. EPHES; cassava,

Key words: Bud dormancy, cell cycle, growth, cyclins.

(Foo et al. 2005; Snowden et al. 2005; Sorefan et al. 2003). Studies on leafy spurge and arabidopsis suggest the auxin signal acts to inhibit cell division and developmental processes in root buds at a point after the G1-to-S phase transition of the cell cycle (del Pozo et al. 2005; Horvath et al. 2002).

Although the primary signals regulating the inhibition of root bud growth have been elucidated (Horvath et al. 2003a), little is known about the cellular processes that respond to these signals or the physiological events that occur to allow growth and development of the growing root buds. Several observations, such as the greening of root buds and the induction of the light-harvesting chlorophyll-binding protein (*LHCB*) gene following loss of paradormancy, have indicated that changes in photomorphogenesis must occur in order for the new shoots to grow (Anderson and Horvath 2000; Anderson et al. 2005; Horvath 1998). Likewise, the induction of genes such as *SHOOTMERISTEMLESS* following loss of paradormancy suggests that other developmental pathways are activated (Varanasi et al. 2005). These “one-gene-at-a-time” approaches based on obvious physiological processes are useful, but they are insufficient for gathering data on the multitude of processes that are likely to occur to both initiate and sustain growth of root buds following loss of paradormancy. Unbiased searches for differentially expressed genes using techniques such as differential display–polymerase chain reaction (PCR) offer some ability to identify unsuspected physiological processes. However, such techniques are often time consuming and expensive because the resulting fragments are often small and thus

require additional cloning and sequencing of longer cDNAs before the putative gene function can be assigned.

Microarray analysis is a powerful method for identifying changes in physiological responses and activation–deactivation of signal transduction pathways following a given treatment. Recent reports have indicated that both heterologous hybridization to either cDNA and long oligonucleotide arrays can be an effective means of identifying tens to hundreds of differentially expressed genes from poorly characterized weed species such as leafy spurge (Horvath et al. 2003b), wild oat (*Avena fatua* L.) (Horvath et al. 2003c), and horseweed [*Conyza canadensis* (L.) Cronq.] (Basu et al. 2005). However, heterologous hybridizations have several limitations, including reduced hybridization specificity and increased potential for cross hybridization between gene family members, and the requirement that hybridizing genes must be cloned and sequenced to confirm their identity. Thus, we have undertaken the development and use of cDNA microarrays consisting primarily of leafy spurge genes to study the developmental processes involved in growth induction of leafy spurge root buds following loss of para-dormancy.

Materials and Methods

Plant Material and RNA Extractions

Plant material, treatments, RNA extraction, and northern blotting procedures used for these experiments have previously been described (Horvath et al. 2002). Briefly, single-stem plants were grown in Sunshine mix¹ in 5- by 21-cm cones in a greenhouse under a 16-h photoperiod with supplemental lighting. Root buds were collected at 0 (control), 12, 24, 48, and 72 h following excision of all plant parts above the base of the crown. Buds for all time points were harvested between 8:00 and 10:00 A.M. (approximately 2 to 4 h after artificial lights were turned on), frozen in liquid nitrogen, and stored at –80 C until RNA extractions were performed. Total RNA was isolated from root buds using a modification (Horvath et al. 2002) of the pine tree extraction method (Chang et al. 1993).

Microarray Design and Hybridization

Microarrays used for these experiments were developed by Centro Internacional de Agricultura Tropical using 1,886 partially sequenced cDNAs (ESTs [expressed sequence tags]) representing 271 contigs and 1,147 singletons produced from a cDNA library of root buds collected from leafy spurge 3 d following excision of the aerial portions of the plant (Anderson and Horvath 2001). It should be noted that such treatment induces growth in only 40 to 60% of the buds and thus the library likely contains genes expressed in both growing and dormant buds (D. P. Horvath, unpublished observations). Additionally, 384 cassava cDNAs and 384 cDNAs from other plant, animal, and bacterial sequences at various concentrations or with non-DNA-containing spotting buffer were included as controls (López et al. 2005). The data discussed in this article, along with detailed information on the microarray design and layout, have been deposited in the National Center for Biotechnology Information's Gene Expression Omnibus (GEO; <http://www.ncbi.nlm.nih.gov/geo/>) and are accessible through

GEO Series accession number GSE2786. All leafy spurge cDNAs were spotted randomly and in triplicate, and the cassava and control cDNAs were spotted randomly and in duplicate, resulting in 7,296 features on the array. Probe preparation and hybridization procedures were done essentially as described by Schaffer et al. (2001). Microarray slides were pretreated by baking at 80 C for 3 h and rehydrated by brief steaming on the slides (by holding over a 70 C water bath). Slides were then cross-linked with ultraviolet light at 60 mJ cm^{–2} using a Hoefer UVC500 cross-linker.² Slides were prehybridized for 5 min at 76 C. Briefly, 40 µl of prehybridization solution was placed on the arrays and a cover slip was added. Prehybridization solution consists of 48% formamide,³ 3.2× SSC, 0.4% SDS,⁴ and 2× Denhardt's solution (see Sambrook et al. 1989 for formulas of SSC and Denhardt's solutions). After prehybridization, the slide and cover slip were placed at an angle upside down in distilled water until the cover slip fell off. Both slide and cover slip were then dipped successively in 70 and 100% ethanol and air dried before hybridization. One hundred micrograms of total RNA was reverse transcribed in the presence of Cy3- or Cy5-labeled dCTP according to Schaffer et al. (2001). Briefly, 15.5 µl of total RNA was mixed with 3 µl of oligo dT₂₃V (2 µg µl^{–1}), heated to 70 C for 10 min, and then cooled on ice. Then 14.5 µl of RT (reverse transcription) cocktail (6 µl 5× Superscript II buffer, 3 µl 0.1 M dithiothreitol, 0.5 µl dNTP mix [25 mM dATP, dTTP, dGTP, and 9 mM dCTP]), 2 µl Cy3- or Cy5-labeled dCTP,⁵ 2 µl Superscript II,⁶ and 1 µl RNasin⁷ (40 U µl^{–1}) were added to each reaction and incubated for 2 to 4 h at 42 C. The reaction was halted and RNA was removed by adding 10 µl of a 0.5 M NaOH/5 mM EDTA solution and heating to 60 C for 20 min followed by neutralizing with 20 µl of a 1 M Tris solution (pH 7.4). The labeled cDNA was purified using a QIAquick PCR Purification Kit⁸ as per the manufacturer's protocol. Labeled cDNA was freeze-dried and resuspended in hybridization buffer (26.4 µl water, 3.75 µl yeast tRNA [2 µg µl^{–1}], 7.65 µl 20× SSC, and 7.2 µl 2% SDS). Hybridization was carried out overnight at 60 C in a humidified chamber as previously described (Horvath et al. 2003b). The resulting arrays were washed according to Schaffer et al. (2001) and scanned on an AFFY428 chip reader.⁹ Feature identification and intensity analysis were accomplished using the Jaguar software package⁹ supplied with the chip reader. A reverse-labeling hybridization scheme was designed to compare two biological replicates for each time point (from 0 to 72 h) to a single standard control sample.

Microarray Data Analysis

Features that produced poor signal intensities in both channels and all blank and nonplant control features were discarded. The remaining feature intensity values were copied and pasted into a MEF file format developed for use in the MIDAS microarray analysis software package developed and provided freely by TIGR (<http://www.tigr.org/software/>). The resulting file was Loess normalized and standard deviation regularized to produce the final hybridization ratios of control vs. treated expression values for each element (feature) on the array. The average and standard deviation of normalized feature ratios for each cDNA was determined (for all six features from leafy spurge clones or four from

TABLE 1. List of genes representing a significant pattern of differential expression. Blue values are those showing a significant pattern of differential expression at the designated time point. P-values determined by Student's *T*-test comparing expression ratios for individual genes versus the expression ratio for all of the genes from array hybridizations at each time point.

Clone ID	Function	Average normalized ratio- control/treated 0-72 hrs					p-value for individual clones vs all clones at given times				
		0h	12hr	24hr	48hr	72hr	0 hr	12 hr	24 hr	48 hr	72 hr
32AX	Asparagine Synthase	0.56	2.63	3.14	1.79	1.66	0.019	0.009	0.000	0.045	0.312
18L	Phosphate Inducible Protein	0.54	2.51	3.80	2.07	1.57	0.020	0.021	0.000	0.004	0.310
p 14 P15	PUTATIVE RIBOSOME-BINDING FACTOR A	0.54	1.52	1.59	2.03	1.37	0.002	0.001	0.004	0.007	0.219
29BD	Unknown	0.60	1.52	1.49	1.22	0.98	0.001	0.004	0.009	0.094	0.485
9AY	Peroxidase	0.51	1.54	1.27	0.86	0.74	0.002	0.032	0.449	0.125	0.000
22AU	Chlorophyll a/b-binding Protein	0.94	1.58	2.50	2.43	1.11	0.541	0.021	0.005	0.000	0.923
20E	Glycoprotein EP1	1.11	2.30	2.39	1.57	1.64	0.914	0.026	0.090	0.006	0.339
35V	Chlorophyll a/b-Binding Protein	1.24	1.55	2.35	2.66	1.32	0.818	0.006	0.007	0.017	0.570
28AX	Chlorophyll a/b-binding Protein	1.29	1.86	1.83	2.15	1.25	0.783	0.000	0.036	0.000	0.639
12ae	Unknown	N/A	1.89	1.69	1.95	2.24	N/A	0.007	0.002	0.014	0.185
23ac	Unknown	0.91	2.82	3.11	2.14	1.35	0.458	0.001	0.000	0.057	0.557
21V	Lectin-Putative	1.45	1.93	1.99	0.86	1.21	0.676	0.046	0.027	0.022	0.519
13Z	Aquaporin (plasma membrane intrinsic protein)	N/A	1.85	1.91	1.27	1.23	N/A	0.025	0.000	0.117	0.273
24aa	Unknown	0.49	1.78	1.87	0.93	N/A	0.095	0.011	0.041	0.195	N/A
6R	NAD-Dependent Formate Dehydrogenase	1.15	1.53	1.84	1.38	1.22	0.926	0.038	0.083	0.014	0.654
35BB1	Calreticulin	N/A	1.60	1.83	1.43	1.26	N/A	0.001	0.003	0.032	0.631
16S	Major Allergen	N/A	2.34	1.81	1.06	0.98	N/A	0.000	0.002	0.962	0.446
15AQ	Unknown	N/A	1.64	1.80	1.02	1.21	N/A	0.002	0.036	0.697	0.288
24P	Hypothetical Protein-Spurg	1.47	1.57	1.72	1.17	0.99	0.741	0.007	0.000	0.426	0.462
13BD	Unknown	0.76	1.61	1.69	1.21	1.18	0.016	0.009	0.002	0.403	0.262
2ak	Unknown	N/A	2.18	1.67	1.56	1.23	N/A	0.002	0.167	0.130	0.690
21BC	Stress-pathogenesis-related Protein	N/A	2.26	1.60	1.14	1.17	N/A	0.002	0.102	0.320	0.540
36AX	Hypothetical Protein-Arab	0.83	1.61	1.58	0.90	0.95	0.056	0.004	0.108	0.011	0.226
26ak	Unknown	1.09	1.59	1.52	0.99	0.90	0.776	0.037	0.050	0.316	0.360
7AT	Photosystem I Reaction Center Subunit VI-like	1.26	1.67	1.50	1.44	1.32	0.706	0.047	0.065	0.004	0.696
24AN	Chlorophyll a/b binding protein	1.76	1.21	1.52	2.27	1.54	0.351	0.459	0.193	0.017	0.215
27L	Chlorophyll a/b-Binding Protein (LHCII Type 1)	1.27	1.18	1.56	2.17	1.55	0.763	0.368	0.141	0.004	0.300
p 14 L21	PUTATIVE RIBOSOME-BINDING FACTOR A	1.14	1.26	1.79	1.87	1.26	0.907	0.188	0.007	0.031	0.114
32AT	Chlorophyll a/b-binding Protein	1.88	1.17	1.72	1.68	1.63	0.131	0.408	0.064	0.023	0.198
23I	Metallothionein-Like	2.01	1.11	2.18	1.67	1.31	0.118	0.742	0.019	0.006	0.026
p 14 M5	60S RIBOSOMAL PROTEIN L32	0.73	1.14	1.51	1.62	1.13	0.009	0.282	0.067	0.043	0.854
p 14 I23	60S RIBOSOMAL PROTEIN L32	0.77	1.32	2.59	1.62	1.42	0.017	0.186	0.169	0.002	0.299
7AX	Shaggy Kinase-like Protein	0.66	1.51	1.58	1.58	0.98	0.020	0.051	0.044	0.007	0.346
35AT	Unknown (Similar to Membrane-related Protein)	0.53	1.67	1.58	1.58	1.05	0.000	0.059	0.000	0.002	0.792
28X	Unknown	0.65	1.39	2.08	1.56	0.43	0.113	0.019	0.003	0.042	N/A
4AV	Hydroxy-Protein-rich Glyco Protein	N/A	1.44	1.51	1.59	1.04	N/A	0.106	0.002	0.000	0.553
ooo11	Unknown	0.94	1.27	1.66	1.53	0.80	0.357	0.116	0.012	0.023	0.043
p 14 M17	60S RIBOSOMAL PROTEIN L32	0.64	1.19	1.53	1.52	1.29	0.079	0.157	0.048	0.004	0.021
32AZ	Cellulase	1.38	0.68	0.57	1.71	0.96	0.259	0.001	0.000	0.019	0.322
30	Gibberellin-Regulated Protein	1.18	0.95	0.53	1.54	1.13	0.900	0.502	0.001	0.037	0.965
14BA	GAS4-like	1.91	0.82	0.50	1.62	1.12	0.109	0.294	0.000	0.006	0.740
31AL	Histone H1-Like Protein	1.99	0.65	0.59	1.62	0.81	0.055	0.001	0.000	0.038	0.006
13S	Histone H2B	2.14	0.62	0.57	1.47	0.92	0.033	0.004	0.000	0.039	0.251
15ab	Unknown	3.42	0.36	0.40	0.59	0.95	0.008	0.000	0.000	0.019	0.792
23AR	Flavone-3-hydroxylase	1.67	0.40	0.44	0.47	0.99	0.501	0.000	0.001	0.000	0.770
9BA	Leucoanthocyanidin Dioxygenase	1.66	0.55	0.54	0.63	1.01	0.058	0.001	0.000	0.006	0.534
2BD	Alcohol Dehydrogenase	2.26	0.30	0.24	0.40	0.14	0.136	0.000	0.000	0.000	0.000
10BB1	Xyloglucan Endotransglycosylase	2.61	0.66	0.85	0.99	0.91	0.012	0.049	0.493	0.462	0.217
3S	Flavone 3 Hydroxylase	1.96	0.63	1.26	0.80	1.20	0.039	0.004	0.708	0.116	0.765
14V	S-Adenosylmethionine Synthase	1.39	0.64	0.60	0.78	1.99	0.587	0.001	0.027	0.004	0.077
24H	Unknown	1.27	0.59	0.57	0.68	1.08	0.735	0.001	0.000	0.003	0.977
17AX	Enolase	1.54	0.59	0.52	0.88	0.90	0.392	0.001	0.000	0.032	0.490
23S	Unknown Protein-Arabidopsis (AC005698)	1.58	0.62	0.48	0.71	0.61	0.360	0.003	0.002	0.001	0.006
5AV	Similar to En/Spm-like Transposon	2.21	0.58	0.45	0.79	0.79	0.054	0.000	0.000	0.022	0.030
3BD	Granule-bound Glycogen [Starch] Synthase	2.01	0.32	0.26	0.69	N/A	0.192	0.000	0.000	0.060	N/A
4bb	Unknown	1.77	1.09	1.77	0.62	1.84	0.212	0.714	0.308	0.003	0.310
5AU	Flavone 3-hydroxylase	1.41	0.71	0.51	0.54	1.42	0.291	0.002	0.000	0.000	0.350
36E	Vegative Storage Protein	0.74	1.95	1.78	0.52	1.45	0.030	0.104	0.116	0.002	0.230
36BD	Rubisco Small Chain	1.04	0.81	0.89	0.47	0.57	0.734	0.017	0.090	0.000	0.001

cassava and control clones from both biological replicates). cDNAs with greater than a 1.5-fold difference in expression and with standard deviations greater than twofold of the average were reinvestigated to determine if the variant features were the result of background or hybridization abnormalities. If the features were shown to be unreliable, the data points from those features were discarded and the ratios were reaveraged for those cDNAs. The remaining data for each clone were subjected to a Student's *t* test to identify clones with significant differential expression by comparing the ratios for all features from any given clone to the ratios of all the features on the array (Supplemental Data Table 1; <http://www.ars.usda.gov/sp2UserFiles/Place/54420510/supplementaldatafile1.xls>). Clustering and tree visualization

were accomplished after log normalization and average linkage clustering using the free software by Eisen (1998).

Northern Hybridization

Total RNA was separated on denaturing agarose gels and blotted according to standard techniques (Sambrook et al. 1989). DNA probes were prepared by PCR amplification of designated cDNAs followed by isolation of the resulting band after separation on agarose gels. Probes for all clones were amplified from previously isolated and sequenced cDNAs using primers developed either specifically for the genes (flavone 3-hydroxylase genes, chalcone synthase, and leucoanthocyanidin oxygenase) or using primers developed

to amplify the inserted cDNA from the cloning vector (all other probes). Probes were radio-labeled with ^{32}P and hybridized to the blots ($5\times \text{SSC}/50\%$ formamide² at 42 °C). Blots were washed four times at room temperature in $2\times \text{SSC}$, 0.2% SDS for 5 min each and then two times at 65 °C for 15 min each. The resulting hybridizations were visualized by autoradiography or on a Packard Instant Imager[®].¹⁰ Linearity was maintained for all of the images presented.

Results and Discussion

Overall Patterns of Differential Expression on Microarray

Microarray analysis was used to identify signaling pathways and putative differentially expressed genes in leafy spurge root buds during the transition from paradormancy to growth. All time points from 0 to 72 h were compared to a single reference sample of paradormant buds, resulting in directly comparable gene expression patterns (GEO series accession number GSE2786). It should be noted that the number of repetitions for these experiments provides insufficient statistical power to convincingly ascertain changes in expression of any individual gene. However, these experiments proved to be sufficient for identifying clusters of coordinately regulated genes indicative of specific changes in identifiable physiological processes. For example, although differential expression of both chalcone synthase and chalcone-flavone isomerase is not statistically significant, some differential expression was observed for these genes (Supplemental Data Table 1; Figure 1). Yet other genes involved in the same pathway (flavone 3-hydroxylase and leucoanthocyanidin oxygenase) showed differential expression at a significant level (Table 1; Figures 1 and 2). Thus we can be confident that signals regulating this physiological response are acting in root buds as they are released from paradormancy. It is equally likely that some genes listed as significantly differentially expressed are false positives, and only clusters of differentially expressed genes known to be coordinately expressed or to act in a common physiological response should be considered likely to represent true changes in gene expression.

The results indicated that the 24-h time point displayed the greatest number of differentially expressed genes. Interestingly, there were also a large number of differentially expressed genes at the 0-h time point. This was surprising because the control sample was also isolated from root buds collected independently at a 0-h time point. It is also noteworthy that none of the significantly differentially expressed genes at 0 h were found to be differentially expressed at a significant level in the same manner (i.e., both up or both down) at 12 h (Table 1). Although the reason for this is not known, there have been unpublished observations that indicate some induction of growth-regulated genes may occur in otherwise paradormant buds. In fact, northern hybridization of the 0-h control and one of the 0-h time points showed substantial expression of *HISTONE H3*, indicating that some uncontrolled environmental or developmental factor may have induced some loss of paradormancy in these samples (Figure 1). However, when the plants were defoliated, the gene expression pattern stabilized, and more genes

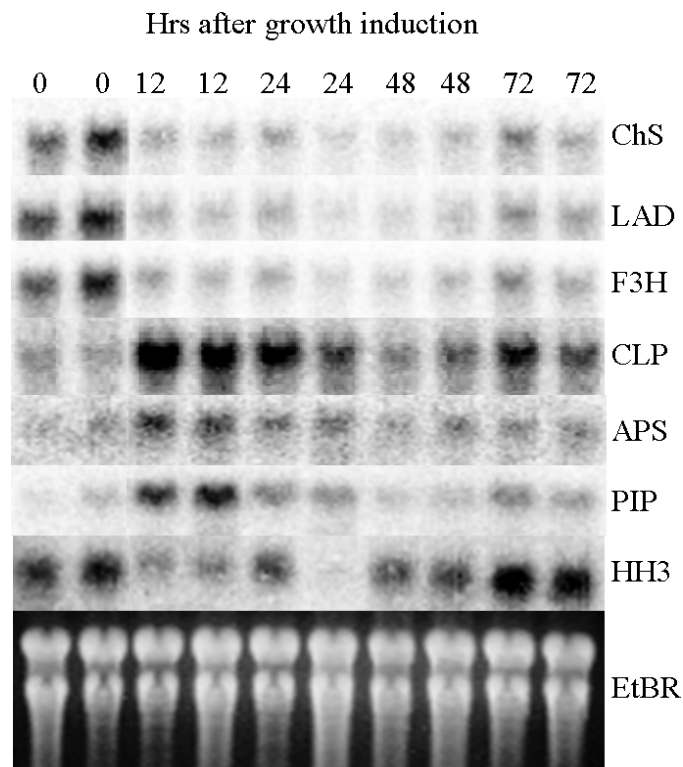


FIGURE 1. Northern analysis of several genes identified as being up- or down-regulated in root buds of leafy spurge following loss of paradormancy. RNA from two independent sets of buds for each time point following defoliation (in hours) was blotted and sequentially hybridized to radio-labeled cDNA fragments from expressed sequence tags (ESTs) representing chalcone synthase (ChS), leucoanthocyanidin oxygenase (LAD), flavone 3-hydroxylase (F3H), curculin-like (mannose-binding) lectin family protein gene (CLP), histone H3 (HH3), asparagine synthase (APS), and phosphate-inducible protein gene (PIP). The ethidium-stained RNA from the blotted gel is shown as a loading control.

were identified that showed consistent patterns of expression through adjacent time points (Table 1).

Signaling Pathways Identified by Cluster Analysis

Cluster analysis was performed on the averaged gene expression data (Figure 2). As expected, signaling pathways regulating cell cycle progression, GA responses, and photomorphogenesis appeared to be activated by defoliation. The observed up-regulation of cell cycle and photomorphogenesis genes following growth induction in root buds has been previously reported and discussed (Anderson and Horvath 2000, 2001; Horvath et al. 2002).

Identification of Genes Expressed Preferentially in Growing Buds

Among other genes that were shown to be significantly up-regulated upon dormancy release was a gene with homology to glycoprotein EP1 in Arabidopsis, which functions as a curculin-like (mannose-binding) lectin family protein similar to S-glycoprotein from birdsrape mustard (*Brassica rapa* L.) (CLP). Primers were designed to amplify this gene from the EST collection, and the resulting fragment was used as a probe for northern blot hybridization (Figure 1). The results of this hybridization indicated that it was up-

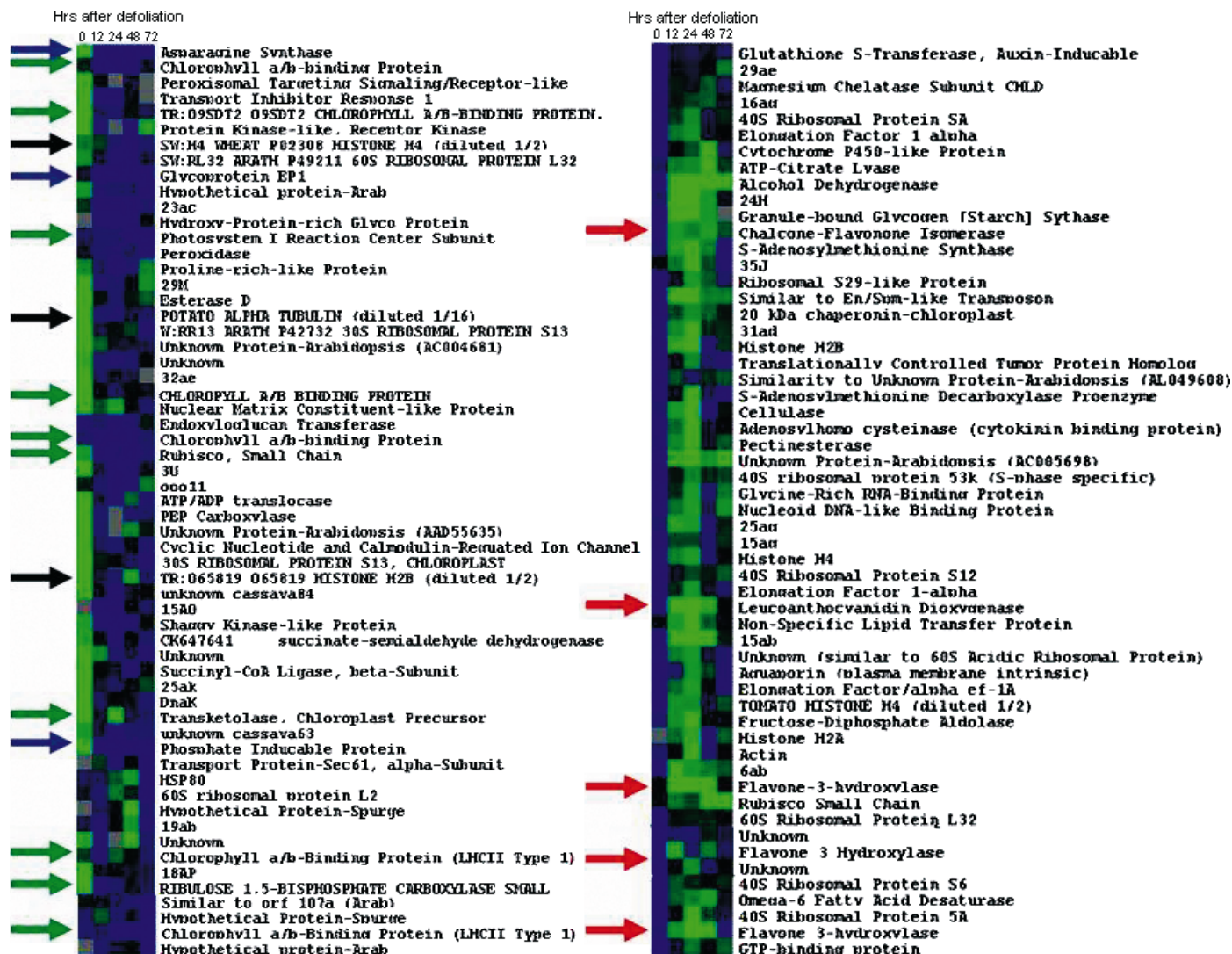


FIGURE 2. Representative sections of the cluster analysis from the normalized ratios of microarray data. Blue indicates increased expression relative to paradormant control tissue, and green represents decreased expression relative to paradormant control tissue. Green arrows point toward genes involved in photomorphogenesis, black arrows point toward genes involved in cell division, purple arrows point toward rapidly induced genes, and red arrows point toward flavonoid biosynthesis genes.

regulated within 12 h following defoliation, thus confirming the microarray data for this gene. Interestingly, the predicted functional category of this gene from Arabidopsis suggests it plays a role in signal transduction processes (http://mips.gsf.de/cgi-bin/proj/thal/search_gene?code=At1g78830). There are five similar genes in the Arabidopsis genome. Two pairs of genes, At1g78820/At1g78830 and At1g78850/At1g78860, are organized as tandem repeats on Chromosome 1 with a single F-box protein gene separating these two pairs, and another similar gene (At1g16900) also located on Chromosome 1 approximately 6.2 Mb away. Expression data are available for three of the five *CLP* genes from Arabidopsis (all expression data for these and other Arabidopsis genes are accessible through the Arabidopsis Information Resource microarray elements link at <http://www.arabidopsis.org/tools/bulk/microarray/index.jsp>). Two of the genes (At1g78850 and At1g78860) appear to be primarily circadian regulated. The third one (At1g78830), which is most similar to the leafy spurge EST, appears to be preferentially expressed in growing and differentiating tissues

(Supplemental Data Table 2; <http://www.ars.usda.gov/sp2UserFiles/Place/54420510/supplementaldatafile2.xls>). Many of the treatments in which At1g78830 is differentially expressed, such as overexpression of *KNOTTED1*, were previously identified as treatments that cause differential expression in other growth-regulated genes from leafy spurge (Horvath et al. 2003b).

Two other consistently and rapidly up-regulated genes (Figure 1) encode an asparagine synthase (*APS*) and a phosphate-inducible protein (*PIP*). Putative orthologues of these genes (At3g47340 and At5g64260, respectively) are also coordinately regulated with At1g78830 in Arabidopsis in response to cytokinin, and both are preferentially expressed in growing shoot tissues of Arabidopsis (Supplemental Data Table 2). The putative Arabidopsis orthologue of *APS* is also suspected of being down-regulated in response to sugar levels (Fujiki et al. 2001). This expression pattern is consistent with previous studies on root buds of leafy spurge that suggest sugar can negatively impact root bud growth (Chao et al. 2006) and that the drop in sugar levels (or loss of pho-

tosynthesis) is perceived by the buds shortly after defoliation (Horvath et al. 2002).

Identification of Genes Expressed Preferentially in Paradormant Buds

Cluster analysis also indicated a general inhibition of pathways involved in flavonoid biosynthesis, as shown by the significant down-regulation of several flavone 3-hydroxylase genes (*F3H*), a chalcone synthase (*ChS*), a chalcone-flavone isomerase (*CFI*), and a leucoanthocyanidin oxygenase gene (*LAD*) (Figure 2). To confirm the differential expression of the flavonoid biosynthesis genes, primers were developed to specifically amplify several of these genes and subsequently used as probes for northern analysis (Figure 1). The results of these experiments clearly indicated that the flavonoid biosynthesis genes encoding *LAD* and *F3H* are down-regulated within 12 h following growth induction. Interestingly, preliminary semiquantitative RT-PCR studies indicate that flavonoid biosynthesis appears to be up-regulated to control levels by 4 to 5 d following growth induction (data not shown). This observation may indicate that differential regulation of these genes, following loss of paradormancy, is due to their response to signals involved in dormancy breaking, but not growth status of the buds.

The down-regulation of flavonoid biosynthesis following growth induction in adventitious buds was previously unknown, and the functional significance of this down-regulation will require further study. There are reports in the literature suggesting that flavones can inhibit auxin transport (Brown et al. 2001; El Euch et al. 1998; Jacobs and Rubery 1988; Murphy et al. 2000). In axillary buds of several species, auxin production increases upon release of buds from paradormancy (Gocal et al. 1991; Stafstrom and Sussex 1988). This increase in auxin production and subsequent transport to the distal regions of the plant results in growth inhibition of distal buds (sometimes referred to as apical dominance). Consequently, one possible hypothesis is that flavonoid biosynthesis needs to be inhibited upon dormancy release to allow polar auxin transport for suppression of growth in more distal buds. This hypothesis is strengthened by observations suggesting polar auxin transport must be initiated before bud elongation (Morris 1977). If this hypothesis is confirmed, it may provide a target mechanism to release all viable buds from dormancy and allow for more effective weed control.

Sources of Materials

¹ Sunshine mix potting soil, Fisons Horticulture Inc., 110 110th Avenue N.E., Suite 490, Bellevue, WA 98004.

² Hoefer UVC500 cross-linker, Amersham Biosciences Corp., 800 Centennial Avenue, P.O. Box 1327, Piscataway, NJ 08855-1327.

³ Formamide (F7508), Sigma-Aldrich Co., P.O. Box 952968, St. Louis, MO 63195.

⁴ Sodium dodecyl sulfate (15525-017), Invitrogen Life Technologies Inc., 1600 Faraday Avenue, Carlsbad, CA 92008.

⁵ Cy3 and Cy5 dCTP, Amersham Biosciences, 800 Centennial Avenue, Piscataway, NJ 08855.

⁶ Superscript II, Invitrogen Corporation, 1600 Faraday Avenue, P.O. Box 6482, Carlsbad, CA 92008.

⁷ RNasin, Promega Corp., 2800 Woods Hollow Road, Madison, WI 53711.

⁸ QIAquick PCR Purification Kit, QIAGEN Inc., 27220 Turnberry Lane, Valencia, CA 91355.

⁹ Affy428 scanner and Jaguar software, Affymetrix, Inc., 3380 Central Expressway, Santa Clara, CA 95051.

¹⁰ Packard Instantimager, Packard Instrument Co., 2200 Warrenville Road, Downers Grove, IL 60515.

Literature Cited

- Anderson, J. V., R. W. Gesch, Y. Jia, W. S. Chao, and D. P. Horvath. 2005. Seasonal shifts in dormancy status, carbohydrate metabolism, and related gene expression in crown buds of leafy spurge. *Plant Cell Environ.* In press.
- Anderson, J. V. and D. P. Horvath. 2000. Isolation of a cDNA clone (Accession # AF220527) encoding a light-harvesting chlorophyll a/b-binding protein (*Lhcb1*) in underground adventitious buds of leafy spurge. *Plant Physiol.* 122:1457.
- Anderson, J. V. and D. P. Horvath. 2001. Random sequencing of cDNAs and identification of mRNAs. *Weed Sci.* 49:590–597.
- Basu, C., M. D. Halfhill, J. N. Burris, B. M. Leckie, T. C. Miller, J. C. Halfhill, and E. N. Stewert. 2005. Towards the understanding of molecular mechanisms of glyphosate resistance in horseweed (*Conyza canadensis*). Abstract # 227 in Weed Science Society of America 45th Annual Meeting Program, Honolulu, HI, p. 40.
- Brown, D. E., A. M. Rashotte, A. S. Murphy, B. W. Tague, W. A. Peer, L. Taiz, and G. K. Muday. 2001. Flavonoids act as negative regulators of auxin transport *in vivo* in *Arabidopsis thaliana*. *Plant Physiol.* 126: 524–535.
- Chang, S., J. Puryear, and J. Cairney. 1993. A simple and efficient method for isolating RNA from pine trees. *Plant Mol. Biol. Rep.* 11:113–116.
- Chao, W. S., M. D. Serpe, J. V. Anderson, R. W. Gesch, and D. P. Horvath. 2006. Sugars, hormones, and environment affect the dormancy status in underground adventitious buds of leafy spurge (*Euphorbia esula*). *Weed Sci.* In press.
- Cline, M. G. 1991. Apical dominance. *Bot. Rev.* 57:318–358.
- Del Pozo, J. C., M. A. Lopez-Matas, E. Ramirez-Parra, and C. Gutierrez. 2005. Hormonal control of the plant cell cycle. *Physiol. Plant.* 123: 173–183.
- Eisen, M. B., P. T. Spellman, P. O. Brown, and D. Botstein. 1998. Cluster analysis and display of genome-wide expression patterns. *Proc. Natl. Acad. Sci. U.S.A.* 95:14863–14868.
- El Euch, C., C. Jay-Allemand, M. Pastuglia, P. Doumas, J. P. Charpentier, P. Capelli, and L. Jouanin. 1998. Expression of antisense chalcone synthase RNA in transgenic hybrid walnut microcuttings. Effect on flavonoid content and rooting ability. *Plant Mol. Biol.* 38:467–479.
- Foo, E., E. Bullier, M. Goussot, F. Foucher, C. Rameau, and C. A. Beveridge. 2005. The branching gene *RAMOSUS1* mediates interactions among two novel signals and auxin in pea. *Plant Cell.* 17:464–474.
- Fujiki, Y., Y. Yoshikawa, T. Sato, N. Inada, M. Ito, I. Nishida, and A. Watanabe. 2001. Dark-inducible genes from *Arabidopsis thaliana* are associated with leaf senescence and repressed by sugars. *Physiol. Plant.* 111:345–352.
- Gocal, G.F.W., R. P. Pharis, E. C. Yeung, and D. Pearce. 1991. Changes after decapitation in concentrations of indole-3-acetic-acid and abscisic-acid in the larger axillary bud of *Phaseolus vulgaris* L-cv. tender green. *Plant Physiol.* 95:344–350.
- Horvath, D. P. 1998. The role of specific plant organs and polar auxin transport in correlative inhibition of leafy spurge (*Euphorbia esula*) root buds. *Can. J. Bot.* 76:1227–1231.
- Horvath, D. P. 1999. Role of mature leaves in inhibition of root bud growth in *Euphorbia esula* L. *Weed Sci.* 47:544–550.
- Horvath, D. P., J. V. Anderson, W. S. Chao, and M. F. Foley. 2003a. Knowing when to grow: signals regulating bud dormancy. *Trends in Plant Sci.* 8:534–540.
- Horvath, D. P., J. V. Anderson, Y. Jia, and W. S. Chao. 2005. Cloning, characterization and expression of growth regulator *CYCLIN D3-2* in leafy spurge (*Euphorbia esula*). *Weed Sci.* 53:431–437.
- Horvath, D. P., W. S. Chao, and J. V. Anderson. 2002. Molecular analysis of signals controlling dormancy and growth in underground adventitious buds of leafy spurge. *Plant Physiol.* 128:1439–1446.
- Horvath, D. P., R. Schaffer, M. West, and E. Wisman. 2003b. Arabidopsis microarrays identify conserved and differentially-expressed genes involved in shoot growth and development from distantly related plant species. *Plant J.* 34:125–134.
- Horvath, D. P., R. Schaffer, and E. Wisman. 2003c. Identification of genes

- induced in emerging tillers of wild oat (*Avena fatua*) using *Arabidopsis* microarrays. *Weed Sci.* 51:503–508.
- Jacobs, M., and P. H. Rubery. 1988. Naturally occurring auxin transport regulators. *Science* 241:346–349.
- López, C., M. Soto-Suárez, S. Restrepo, B. Piegu, R. Cooke, M. Delseny, J. Tohme, and V. Verdier. 2005. Global transcriptome analysis of cassava responses to *Xanthomonas axonopodis* pv. *manihotis* infection using a cassava cDNA microarray. *Plant Mol. Biol.* In press.
- Morris, D. A. 1977. Transport of exogenous auxin in two-branched dwarf pea seedlings (*Pisum sativum* L.): some implications for polarity and apical dominance. *Planta* 136:91–96.
- Murphy, A., W. A. Peer, and L. Taiz. 2000. Regulation of auxin transport by aminopeptidases and endogenous flavonoids. *Planta* 211:315–324.
- Sambrook, J., E. F. Fritsch, and T. Maniatis. 1989. *Molecular Cloning*. 2nd ed. New York: Cold Spring Harbor Laboratory Press. Pp. B.13 and B.15.
- Schaffer, R., J. Landgraf, M. Accerbi, V. V. Simon, M. Larson, and E. Wisman. 2001. Microarray analysis of diurnal and circadian-regulated genes in *Arabidopsis*. *Plant Cell* 13:113–123.
- Snowden, K. C., A. J. Simkin, B. J. Janssen, et al. 2005. The *Decreased apical dominance1/Petunia hybrida CAROTENOID CLEAVAGE DIOXYGENASE8* gene affects branch production and plays a role in leaf senescence, root growth, and flower development. *Plant Cell* 17: 746–759.
- Sorefan, K., J. Booker, K. Haurogné et al. 2003. *MAX4* and *RMS1* are orthologous dioxygenase-like genes that regulate shoot branching in *Arabidopsis* and pea. *Genes Dev.* 17:1469–1474.
- Stafstrom, J. P. and I. M. Sussex. 1988. Patterns of protein-synthesis in dormant and growing vegetative buds of pea. *Planta* 176:497–505.
- Varanasi, V., Y. Jia, W. S. Chao, J. V. Anderson, and D. P. Horvath. 2005. Understanding shoot development and growth in weeds: cloning and expression of *SHOOTMERISTEMLESS* from leafy spurge. Abstract # 224 in *Weed Science Society of America 45th Annual Meeting Program*, Honolulu, HI, p. 40.

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